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(54) High sensitive assay method of L-carnitine and composition for an assay.

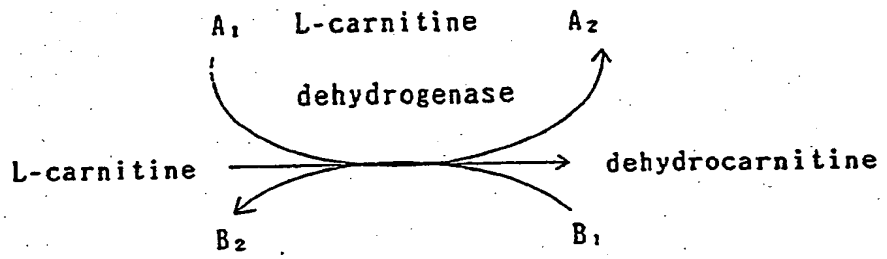
(57) A method of assaying L-carnitine which comprises reacting a specimen comprising the L-carnitine to be assayed with reagents comprising :

① L-carnitine dehydrogenase which has coenzymes of the thionicotinamide adenine dinucleotide group (hereinafter designated as the thio-NAD-group) and nicotinamide adenine dinucleotide group (hereinafter designated as the NAD group) and which catalyses a reversible reaction forming essentially dehydrocarnitine from L-carnitine ;

② A_1 ; and

③ B_1 ;

to perform the cycling reaction :



wherein :

A_1 is the thio-NAD group or the NAD group ;

A_2 is a reduced form of A_1 ;

when A_1 is the thio-NAD group, B_1 is a reduced NAD group and when A_1 is the NAD group, B_1 is a reduced thio-NAD group, and

B_2 is an oxidized form of B_1 ;

and measuring the amount of A_2 generated or B_1 consumed.

EP 0 437 373 A1

HIGH SENSITIVE ASSAY METHOD OF L-CARNITINE AND COMPOSITION FOR AN ASSAY

Field of the Invention

This invention relates to a high sensitive assay method of L-canitine which is especially L-carnitine in a specimen, and a composition for assaying L-carnitine.

The prior arts

L-carnitine is an essential substance for mediating a long-chain fatty acid transport through mitochondrian membrane prior to intracellular β-oxidation, and hence a deficiency of L-carnitine causes disorders in fatty acid and its related metabolisms. Particularly, it is believed that disorders of the skeletal muscle and cardiac muscle, both of which are high energy consumption tissues depending on cartinine and lacking in carnitine generation, occur from such deficiency. Heretofore a desease arising from inborn irregularities of carnitine metabolism has been studied, however in recent time, secondary disorders of carnitine metabolism have become a problem in patients suffering from nephrosis and undergoing dialysis. Carnitine is administered to carnitine deficient patients who have a disease of the body muscle or cardiacmuscle, or patients undergoing dialysis. Studies on the behavior of carnitine in disease and therapy have been requied, however a desirable assay method for carnitine in the clinical field has not been developed.

known assay methods for carnitine are as follows:

1. L-carnitine and acetyl CoA are treated with carnitine acetyltransferase (CAT), and the thus-liberated CoASH and 5, 5'- dithio-bis-2-nitrobenzoate (DTNB) are further reacted to generate thiophenolate ion which is colorimetrically measured (DTNB method). This method is described in J. Biol. Chem., Vol.238, p. 2509 (1963), J. Lipid Res., Vol. 5, pp. 184-187 (1964) and Clinical Pathology, Vol. 36, N. 11, pp. 1296-1302 (1988).

2. L-carnitine and ^{14}C - or ^{3}H - labelled acetyl CoA are treated with CAT to generate labelled acetyl-L-carnitine and CoASH, and radio-activity is measured (radioisotope method). This method is described in Clin. Chem. Acta, Vol. 37, pp. 235-243 (1972), J. Lipid Res., Vol. 17, pp. 277-281 (1976), and J. Japan. Nut. Food. Soc., Vol. 41, N. 5, pp. 389-395 (1988).

3. L-carnitine and NAD^+ are treated with L-carnitine dehydrogenase to generate 3-dehydrocarnitine and NADH, and increased UV absorption of NADH is measured (carnitine dehydrogenase method). This method is described in Eur. J. Biochem., Vol. 6, pp. 196-201 (1968), ibid. Vol. 10, pp. 56-60 (1969), and Fresenius Z. Anal. Chem., Vol. 320, N. 3, pp. 285-289 (1985).

4. L-carnitine and acetyl CoA are treated with CAT to generate CoA which is reacted with n- {p-(2-benzimidazolyl)-phenyl } -malimide (BIPM), and fluorescent intensity of the resulting CoA-BIPM is measured (fluorescence method). This method is described in Ann. Rep. MHW Institute for Nerve Disease, pp. 315-318 (1986).

Problems to be solved by the invention

In the prior arts, the DTNB method and the fluorescence method are essentially required a treatment of deproteinization in an assay of serum L-carnitine, which causes a complex operation, and radioisotope method has an advantages is its sensitivity and specificity, however special facilities are required for measuring radioactivity. The carnitine dehydrogenase method has disadvantage due to small molecular absorption coefficient of NADH, i.e. $\varepsilon = 6.22$ (cm^2/μ mol) at 340 nm, and hence it is difficult to assay serum carnitine in a patient of carnitine deficient disease [Neurology, 25 : 16-24(1975)], moreover generated NADH is consumed by another dehydrogenase in serum such as lactate dehydrogenase, which causes an error in measurement.

We had invented previously an assay method of L-carnitine, in which generated formazane in an enzymatic reaction with L-carnitine dehydrogenase was quantitatively measured (Japan.Pat. Appln. No 1-196550). The said methods has, however, disadvantages with having insufficient sensitivity for a small amount of serum which being collectd from prematured infant such as an amount of 20 μl as compared with relatively large amount of serum from adult.

Under these circumstances, it has been desired to develop advantageous method for assaying L-carnitine without required complex treatment such as deproteinization and specific facilities and with having possibility for measuring trace amont of serum L-carnitine of prematured infant.

Description of the invention for solving the problem

We have studied a reaction system using L-carnitine dehydrogenase (EC 1.1.1.108), and found that, in a

reversible reaction in which dehydrocarnitine was generated from substrate L-carnitine, when a reaction system, wherein dehydrocarnitine was generated from L-carnitine with a coenzyme of NAD group, together with another trac amount NADH group of coenzyme, was subjected to reversible cycling reaction between L-carnitine and dehydrocarnitine, a linear increase of generated amount of NADH group was observed depending on a time course, furthermore an increasing rate thereof was in proportion to an amount of L-carnitine or dehydrocarnitine.

We have further found that in the said enzymatic cycling reaction, thionicotinamide adenine dinucleotide group (hereinafter desigantes as thio-NAD group) or reduced from thio-NAD group (hereinafter designates as thio-NADH group) is used in NAD group or NADH group, and an amount of changes in any of coenzymes is measured depending upon a difference in a maximum absorption of NADH group at approx. 340 nm and that of thio-NADH group at approx. 400 nm, an amount of L-carnitine or dehydrocarnitine can precisely be measured.

Objects of the Invention

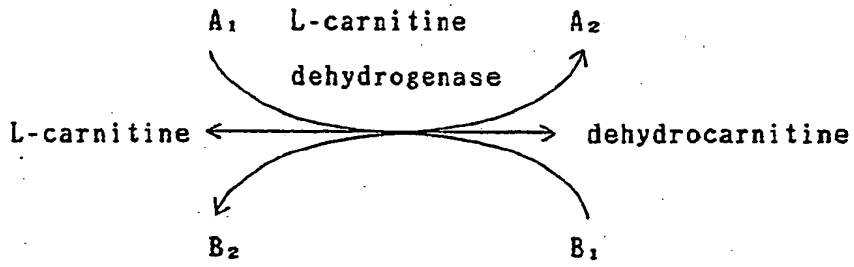
An object of the present invention is to provide a high sensitive assay method of L-carnitine which comprises reacting a specimen with reagents containing

①L-carnitine dehydrogenase which has coenzymes of thionicotinamide adenine dinucleotides group (hereinafter designates as thio-NAD group) and nicotinamide adenine dinucleotides group (hereinafter designates as NAD group) and which catalyses a reversible reaction with forming essentially dehydrocarnitine from a substrate L-carnitine,

②A_1 and

③B_1

to construct a cycling reaction of the formula



wherein A_1 is thio-NAD group or NAD group, A_2 is a reduced form of A_1, when A_1 is thio-NAD group, B_1 is reduced NAD group and when A_1 is NAD group, B_1 is reduced thio-NAD, and B_2 is oxidized form of B_1 and measuring an amount of A_2 or B_1 which depends on the said reaction.

Another object of the present invention is to provide a composition for assaying L-carnitine which comprises containing essentially the following components ① - ③

①L-carnitine dehydrogenase which has coenzymes of thionicotinamide adenine dinucleotides group (hereinafter designates as thio-NAD group) and nicotinamide adenine dinucleotides group (hereinafter designates as NAD group) and which catalyses a reversible reaction with forming essentially dehydrocarnitine from a substrate L-carnitine,

②A_1 and

③B_1

wherein A_1 is thio-NAD group or NAD group, when A_1 is thio-NAD group, B_1 is reduced form of NAD group, and when A_1 is NAD group, B_1 is reduced form of thio-NAD group.

Brief Description of the Drawings

Figure 1 : Rate assay on an amount of L-carnitine at 400 nm in Example 1.

Figure 2 : Rate assay on an amount of serum at 400 nm in Example 2.

In the present invention L-carnitin dehydrogenase can be used in any type of the said enzyme having the properties hereinabove. Examples of L-carnitine dehydrogenase used in the present invention are L-carnitine dehydrogenase produced by th following microorganisms.

Pseudomonas aeruginosa A 7244 (NCTC) [Eur. J. Biochem., Vol. 6, pp. 196-201 (1968), ibid., Vol. 10, pp.

56-60 (1969)] ;
Pseudomonas putida IFP 206 (Arch. Microbiol., Vol. 116, pp. 213-220 (1978), Biochem. Biophys. Acta, Vol. 957, pp. 335-339 (1988) ;
Pseudomonas putida ATCC 17633 [Fresenius' Z. Anal. Chem., Vol. 320, pp. 285-289 (1985)] ; and
Xanthomonas translucens IF0 13558 [Agr. Biol. Chem., Vol. 52, pp. 851-852 (1988)] .
Alcaligenes sp. No. 981 FERM BP-2570 [Product of Toyo Jozo Co., Japan. Pat. Appln. No. 1-267919]
Among these, L-carnitine dehydogenase originated from Alcaligenes sp. No. 981 is preferable in its stability in a buffer solution.

L-carnitine dehydrogenase originated from Alcaligenes sp. No. 981 is a novel L-carnitine dehydrogenase, which is produced, for example, by a micoroorganisms of the genus Alcaligenes sp. No. 981 FERM BP-2570, isolated from a soil sample from a potate field in Gojo-shi, Nara prefecture, Japan.

Detailed Description of the Invnetion

The toxonomical properties of this strain are illustrated as follows :

A. Morphological properties :

Observations on a nutrient agar medium, cultures for 18-24 hours at 28-30°C, are as follows.
Round edge with straight or slightly curved bacillus and single or double linked somewhat short chain. No formation of spores. Sizes are 0.4-0.6 X 1.2-2.5 μm. Peritrichal movement. No polymorphism.

B. Growth on various media :

Observations on various media, cultured for 18-24 hours at 28-30 °C, are as follows.

1. Nutrient agar slant medium :

Good growth with filiform.
Wettish with luminescence. Ocherous but no formation of soluble pigment.

2. Nutrient agar plate medium :

Round, convex and whole round colonies. Smooth wettish surface. Ocherous or pale ocherous.
No formation of soluble pigment.

3. Liquid medium (Aqueous peptone) :

Good growth with uniform turbidity. Formation of pellicle at long term (over 40 hours) culture.

4. BCP milk medium :

Alkaline after 4-5 days.

C. Physiological properties (+ = positive, (+)= weekly positive, − = negative)

Gram-strain	−
KOH reaction	+
Capsule formation	−
Acid fastness stain	−
OF-test (Hugh Leifson)	No change
OF-test (nitrogen source: $NH_4H_2PO_4$)	O (oxidative)
Aerobic growth	+

Anaerobic growth		−
Growth temperature	41°C	−
	37°C	+
	15°C	+
Halotolerant NaCℓ conc. %		
	0 %	+
	5 %	+
	7 %	−
Growth pH	pH 4.6	−
	pH 5.4	+
	pH 8.9	+
	pH 9.8	−
Gelatin hydrolysis		−
Starch hydrolysis		−
Casein hydrolysis		−
Esculin hydrolysis		−
Cellulose hydrolysis		−
Tyrosine hydrolysis		−
Catalase production		+
Oxidase production		+
LV-reaction		−
Urease production (SSR)		−
Urease production (Chris)		−
Indol production		−

H_2S production (detection: lead acetate paper)	−
Acetoin production (K_2HPO_4)	−
Acetoin production (NaCl)	−
MR test	−
Nitrate reduction	
Gas detection	+
NO_2^- detection	−
NO_3^- detection	−
Utilization on Simmons medium	
Citrate	+
Malate	+
Maleate	−
Malonate	(+)
Propionate	−
Gluconate	−
Succinate	+
Ultization on Christenssen medium	
Citrate	+
Malate	+
Maleate	+
Malonate	+
Propionate	−

Gluconate	+
Succinate	+
Gas production from glucose	−
Acid formation from sugar	
Adonitol	−
L(+) arabinose	(+)
Cellobiose	−
Dulsitol	−
Meso-erythritol	−
Fructose	−
Galactose	+
Glucose	+
Glycerin	(+)
Inositol	−
Inulin	−
Lactose	−
Maltose	−
Mannitol	−
Mannose	+
Melezitose	−
Melibiose	−
Raffinose	−
L(+) rhamnose	−
D-ribose	−
Salicin	−
L-sorbose	−

Sorbitol	—
Starch	—
Saccharose	—
Xylose	—
Trehalose	—
Poly- β-hydroxybutyrate accumulation	—

D. Utilization of carbon sources :

Test medium : liquid medium (pH 7.0) containing carbon source 5 g, NaCl 5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $NH_4H_2PO_4$ 1.0 g and distilled water 1 ℓ it. Results are as follows :

Glucose	+
L(+) arabinose	—
Fructose	+
Mannitol	—
Mannose	+
Gluconate	+
Acetate	+
Adipate	—
Pimerate	+
Suberate	+
Tartrate	+

According to the above taxonomical properties, the microorganism displays the specific characteristics of Gram negative bacillus, namely, it is peritrichal in movement, is catalase positive and oxidase positive, does not produce acid from glucose in Hugh-Leifson medium containing pepton, and promotes oxidative decomposition of glucose and acid formation. It displays no spore formation nor polymorphism, and is aerobic.

Among Gram-negative bacillus, there are three microorganisms genera which are peritrichal in movement, namely Alcaligenes, Chromobacterium and Flavobacterium. Chromobacterium produces violet colored pigment, and Flavobacterium produces yellow colored pigment ; however, the present strain does not produce pigment. Hence the present strain belongs to the genus Alcaligenes.

Taxonomic properties of Alcaligenes in comparison with those of the present strain according to Bergey's Manual of Systematic Bacteriology, Vol. 1 (1984), are illustrated with comparing Alcaligenes faecalis (hereinafter designated as F), Alcaligenes denitrificans subsp. denitrificans (hereinafter designated as D) and Alcaligenes denitrificans subsp. xylosoxidans (hereinafter designated as X), as follows :

+ = positive probability over 90 % .
— = negative probability over 90 % .
d = not identified as + or—.

	F	D	X	The present Strain
Oxidase production	+	+	+	+
Nitrate reduction	−	+	+	+
Nitrite reduction	+	+	+	+
Gelatin hydrolysis	−	−	−	−
Acid formation in OF-medium				
Xylose	−	−	+	−
Glucose	−	−	+	−
Acid formation in peptone-free medium				
Xylose			+	−
Glucose			+	+
Utilization of carbon sources				
Glucose	−	−	+	+
L(+) arabinose	−	−	−	−
Fructose	−	−	d	+
Mannitol	−	−	−	−
Mannose	−	−	d	+
Gluconate	−	+	+	+
Acetate	+	+	+	+

According to the above comparison, the present strain No.981 has many identical properties with Alcaligenes denitrificans subsp. xylosoxidans but has specific differences as to acid formation in OF-medium and acid formation from xylose. Accordingly, the present strain has been designated Alcaligenes sp. No. 981 and has been deposited at Fermentation Research Institute and assigned No. FERM BP-2570.

In the enzymatic reaction hereinbefore illustrated, A_1 or B_2 is thio-NAD group or NAD group of coenzymes. Examples of NAD group are nicotinamide adenine dinucleotido (NAD), acetylpyridine ad nine dinucleotide (acetyl NAD), acetylpyridine hypoxanthine dinucleotide and nicotinamid hyp xanthine dinucleotide (deamino NAD). Examples of thio-NAD group are thionicotinamide adenine dinucleotid (thio-NAD) and thionicotinamide hypoxanthine dinucleotide

In the present invnetion, when A_1 is thio-NAD group, B_1 is NAD group, and when A_1 NAD group, B_1 is thio-NADH. Hence at least the one should be thio-type coenzyme.

Amounts of A_1 and B_1 is being excess as compared with that of L-carnitine and is essentially being excess

as compared with Km-value of carnitin dehydrogenase for A_1 and B_1, and specifically 20-10,000 molar excess of an amount of carnitine is preferable.

In a composition for assay of L-carnitine of th present invention, concentration f A_1 and B_1 is 0.02-100 mM, preferably 0.05-30 mM, and a concentration of L-carnitine dehydrogenase is 5-1000 U/ml, preferably 10-150 U/ml, or more.

L-carnitine dehydrogenase used in the composition for assay of L-carnitine of the present invention can be an enzyme having reactivity on a substrate L-carnitine together with preferable coenzyme NAD or thio-NAD. It can be confirmed by using the said coenzyme and substrate. L-carnitine dehydrogenase produced by Alcaligenes sp. No. 981 (product of Toyo Jozo) has relative activity of approx. 15 % when coenzyme thio-NAD is used as compared with NAD. Km-value on L-carnitine, NAD and thio-NAD at the same condition is 9.3 mM, 0.14 mM and 0.40 mM, respectively.

In a composition of reaction medium, two kinds of coenzymes are selected by considering relative activity of L-carnitine dehydrogenase on an each coenzyme, thereafter pH condition thereof on each optimum pH of the forward reaction and reverse reaction is adjusted to setup the pH-condition wherein a ratio of reaction rate on the forward reaction and reverse reaction reaches to 1.

In the present invention, L-carnitine dehydrogenase from single origin or plural origins can be used.

L-carnitine in a specimen can be assayed by adding a specimen 0.001-0.5 ml to the composition for assay containing the above components ① - ③, reacting at 37°C, then measuring an amount of generated A_2 or consumed B_1 on an interval of the two points after starting the reaction, for example one minute between 3 minutes and 4 minutes after starting, or 5 minutes between 3 minutes and 8 minutes after starting the reaction, by determining the changes of absorption at each optical absorption. Alternatively, an enzymatic reaction is stopped after constant time of starting the reaction, for example after 10 minutes, then changes of absorption value can be measured. For example, in the case that A_2 is thio-NADH and B_1 is NADH, a generating A_2 is measured by an increase of absorption at 400 nm [molecular absorption coefficient : 11,200 $M^{-1}cm^{-1}$ (Methods in Enzymolgy. Vol. 55, p. 261 (1979)] or a consuming B_1 is measured by a decrease of absorption at 340 nm (molecular absorption coefficient : 6220 $M^{-1}cm^{-1}$), then the thus obtained value is compared with the value of known concentration of authentic L-carnitine, thereby a concentration of L-carnitine in a specimen can be measured with real-time.

According to an assay method of the present invention, since L-carnitine per se in a specimen is introduced into an enzymatic cycling reaction, it is hard to be effected with coexisting substance in the specimen, and hence a measurement of blank value of specimen is not reqired. Therefore simple assay system by a rate assay can be achieved.

In the present invention, measuring a value of A_2 or B_1 by absorbancy can be replaced by the other known enzymatic methods.

Further in the assay method of the present invention, not only a free L-carnitine but also a liberated L-carnitine from hydrolysis of acyl carnitine such as acetyl carnitine can also be assayed. L-carnitine per se in a specimen can directly be assayed by an assay method of the present invention without hydrolysis of the specimen. Thereafter total amount of L-carnitine and acylcarnitine can be assayed after hydrolysing the specimen. An amount of acylcarnitine in the specimen can be obtained by that an amount of L-carnitine in the specimen without hydrolysis is reducted from a total amount of L-carnitine after hydrolysing acylcarnitine in the specimen.

Furthermore, a substrate in an enzymatic reaction system on a generation or consumption of L-carnitine, or an enzymatic activity thereof can also be assayed. Examples of these enzymatic systems are :

$$\gamma\text{-butyrobetaine} + \text{2-oxoglutarate} + O_2 \xrightarrow{\gamma\text{-butyrobetain-hydroxylase (EC 1.14.11.1)}} \text{L-carnitine} + \text{succinate} + CO_2$$

$$\text{acetylCoA} + \text{L-carnitine} \xrightleftharpoons{\text{carnitine-acetyl transferase (EC 2.3.1.7)}} \text{CoASH} + \text{O-acetylcarnitine}$$

$$\text{O-acylcarnitine} + H_2O \xrightarrow{\text{acylcarnitine-hydolase (EC 3.1.1.28)}} \text{fatty acid} + \text{L-carnitine}$$

$$\text{carnitine} \xrightarrow{\text{carnitine dercarboxylase (EC 4.1.1.42)}} \text{2-methylcholine} + H_2O$$

and in these systems, measurement of substrate or enzymatic activity can be achieved by the assay method of the present invnetion.

Effect of the Invention

As hereinabove explained, the present invention has advantages that no error on measurement can not be occured due to use coenzyme having each different absorption in its reduced form, and that free L-carnitine, total carnitine and acylcarnitine can also be assayed with precisely and rapidly even by a small amount of specimen.

Examples

The following examples illustrate the present invention but are not to be construed as limiting.

Ref. Example 1

(i) Culturing Alcaligenes sp. No. 981:

DL-carnitine hydrochloride (Sigma Chem., Co.)	3.0 %
KH_2PO_4	0.2 %
K_2HPO_4	0.4 %
$MgSO_4 \cdot 7 H_2O$	0.05 %
$FeSO_4 \cdot 7 H_2O$	0.002%
$MnSO_4 \cdot nH_2O$	0.001%
pH 7.0	

100 ml of a liquid medium comprising the above composition was sterilized in a 500 ml Erlenmeyer flask was sterilized at 120°C for 20 minutes. One loopful of Alcaligenes sp. No. 981 was inoculated into the medium and the medium was cultured at 28 °C with stirring at 120 rpm for 40 hours to obtain the cultured mass (95 ml)(enzyme activity : 1.2 U/ ml).

(ii)

DL-carnitine hydrochloride (Sigma Chem. Co.)	3.0 %
yeast extract (Kyokuto Seiyaku Co.)	0.1 %
K_2HPO_4	0.054%
KH_2PO_4	0.746%
$MgSO_4 \cdot 7 H_2O$	0.05 %

$CaCl_2 \cdot 2H_2O$	0.002%
$FeSO_4 \cdot 7H_2O$ (pH 7.0)	0.002%
$MnSO_4 \cdot nH_2O$	0.002%
disform CB 442 (Nihon Yushi Co.)	1 ml /lit.
pH 7.0	

20 l of a liquid medium comprising the above composition was sterilized in a 30 l jar fermenter by heating. 90 ml of the precultued seed culture obtained in step (i) above was inoculated therein and the mixture was cultured at 28 °C, with aeration of 20 l /min, inner pressure 0.4 kg/cm^2, and agitation at 200 rpm for 27 hours to obtain the cultured masss (19 l).(enzyme activity : 3.0 U/ml).

Ref. Example 2

Purification of enzyme :

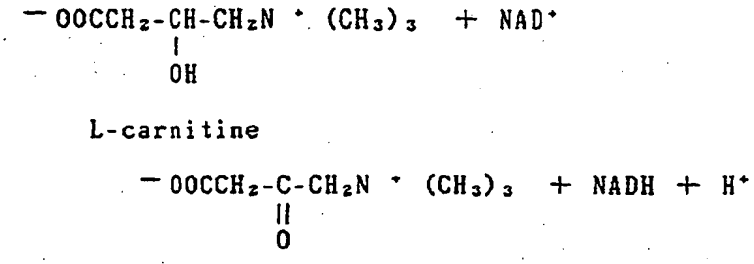
Bacterial cells collected by centrifugation from the cultured broth (19 ℓ) obtained in Ref. Example 1, Culture (ii), were suspended in 40 mM Tris-HCl buffer (pH 8.0, 5 l) containing 0.1 % lysozyme and 15 ml EDTA·2 Na and solubilized at 37°C for 1 hour ; then the mixture was centrifuged to remove precipitate and to obatin a supernatant soulution (4500 ml)(activity : 10.3 U/ml). 1100g ammonium sulfate was added to the supernatant solution, which was mixed well by stirring and then centrifuged to separate the precipitate. An additional 700 g ammonium sulfate was then added to the supernatant solution to dissolve the precipitate, and the solution was centrifuged to obtain a new precipitate. The new precipitate was dissolved in 40 mM Tri-HCl buffer (pH 8.0, 500 ml)(specific activity 84.1 U/ml), and the resultant solution was dialysed against 40 mM Tris-HCl buffer (pH 8.0, 10 lit.). The dialyzed enzyme solution was charged on a column of DEAE-Sepharose CL-6B (Pharmacia Co.) (200 ml) which was buffered with 40 mM Tris-HCl buffer (pH 8.0), washed with 40 mM Tris-HCl buffer containing 0.1 M KCl, (pH 8.0, 1 lit.) and eluted with 40 mM Tris-HCl buffer containing 0.3 M KCl (pH 8.0) to obtain an enzyme solution (300 ml, specific activity 120.5 U/ml). The enzyme solution was dialysed against 40 mM Tris-HCl buffer (pH 8.0, 10 lit.). The Dialysed enzyme solution was charged on a column of hydroxylapatite (KOKEN Co., 100 ml), which was buffered with 40 mM Tris-HCl buffer, washed with 40 mM Tris-HCl buffer, washed with 40 mM Tris-HCl buffer (pH 8.0, 200 ml), then eluted with 2 mM phosphate buffer (pH 7.0, 100 ml) to obtain enzyme solution (100 ml, specific activity 331 U/ml). The thus obtained enzyme solution was dialysed against 20 mM phosphate buffer (pH 7.5, 5 lit.) to obtain 95 ml of an enzyme solution having a specific activity of 331 U/ml. The yield was 67.8 %.

The purified L-carnitine dehydrogenase was found to have an NADH oxidase activity of less than 0.0001 U/ml.

The L-carnitine dehydrogenase thus obtained has the following properties :

1. Enzyme action :

The enzyme catalyses at least a reaction of L-carnitine and NAD $^+$ to generate 3-dehydrocarnitine and NADH, as shown below.



L-carnitine

3-dehydrocarnitine

2. Substrate specificity :

L-carnitine	100 %
Choline	0
Glycinebetaine	0
Glucose	0
Lysine	0

3. Molecular weight :

51000 ± 6000
Measured by TSK-gel G3000 SW (Toso Co., 0.75 X 60 cm)
Elution : 0.1 M phosphate buffer (pH 7,0) containing 0,2 M NaCl.
Standard : following molecular markers (Oriental yeast Co.) are used.

M. W. 12,400	Cytochrome C
M. W. 32,000	adenylate kinase
M W. 67,000	enolase
M. W. 142,000	lactate dehydrogenase
M. W. 290,000	glutamate dehydrogenase

4. Isoelectric point :

pH 5.3 ± 0.6
Measured by electrofocussing using carrier ampholite at 4 °C, 700V, for 40 hours. The activity of a fraction of each enzyme is measured.

5. Km-value : 0.141 mM (NAD^+), 9.3 mM (L-carnitine)

Km-value for NAD^+ is measured in various concentrations of NAD^+ in a reaction mixture of :
100 mM Tris-HCl buffer (pH 9.0)
5 U diaphorase (Toyo Jozo Co.)
0.025 % NBT (Wako Pure Chem. Co.)
1 % polyoxyethylene (20) sorbitan monooleate 80 (Wako Pure Chem., Co.) and
50 mM L-carnitine.
In the reaction mixture, 50 mM L-carnitine is replaced by 1 mM NAD^+, and concentration of L-carnitine is varied to measure the Km-value of L-carnitine.
The results are as shown above.

6. Heat stability :

The enzyme, dissolved in 20 mM Tris-HCl buffer (pH 8.0), to produce a 1.00 U/ml solution, is incubated for one hour at various temperatureas, and the residual activity is measured.
The results can be seen that the enzyme is stable up to 45°C .

7. Optimum temperature

The enzyme activity is measured at 35, 40, 45, 50, 55 and 60°C, respectively, in 100 mM Tris-HCl buffer (pH 9.0) according to the assay method illustrated hereinafter. The reaction was stopped in each case after 10 mins. incubation by adding 0.1 N HCl (2 ml), whereupon the optical absorption at 550 nm. The enzyme shows maximum activity at 50°C.

8. pH - stability :

The residual activity of the enzyme (1 U/ml, 40 mM buffer solution) is measured in various buffer solutions, i.e. acetate buffer, pH 5.6-6.0 ; phosphate buffer, pH 6.0-8.0 ; Tris-HCl buffer, pH 8.0-9.0 and glycine-NaOH buffer, pH 9.0-10, after heating at 45 °C for 30 mins. The enzyme is stable at pH 8.0-9.0 with a residual activity of over 95 %.

9. Optimum pH :

In an assay method for enzyme activity as illustrated hereinafter, 100 mM Tris-HCl buffer in the r action mixture is replaced by 100 mM phosphate buffer (pH 6.5-7.5), 100 mM Tris-HCl buffer (pH 8.0 -9.0) and 100 mM glycine-NaOH buffer (pH 9.0-10.0), and incubated at 37°C for 10 mins. The reaction was stopped in each case by adding 0.1N HCl (2 ml), whereupon the absorption at 550 nm was measured.

A maximum activity is observed at approx. pH 9.0.

10. Long term stability in aqueous solution :

Stability of L-carnitine dehydrogenase added with 0.05 mM NAD is measured in 50 mM Tris-HCl buffer (pH 9.0, 10 U/ml) at 5 °C after two weeks storage.

L-carnitine dehydrogenase of the present invention has a residual activity of 96 % after one week and 82 % after two weeks, thus showing superior stability.

11. Assay method of L-carnitine dehydrogenase activity :

(1) Reaction mixture :

50 mM Tris-HCl buffer (pH 9.0)
1 mM NAD +
5 U Diaphorase (Toyo Jozo Co.)
0.05 % NBT (Wako Pure Chem. Co.)
100 mM KCl
0.5 % polyoxyethylene (20) sorbitan monooleate (Wako Pure Chem. Co.)
100 mM L-carnitine (Sigma Chem. Co.)

(2) Enzyme assay :

The above reaction mixture (1 ml) is incubated in a small test tube at 37 °C for 5 mins. Dilute enzyme solution (0.02 ml) is added and stirred to initiate the reaction. After exactly 10 mins., 0.1 N HCl (2.0 ml) was added and stirred to stop the reaction. Absorption at 550 nm (A_{550} nm) is measured to obtain absorption A_1. The assay was repeated using the above reaction mixture except that L-carnitine was not included. The mixture is also treated in the same manner as described above and its absorption A_0 was measured.

(3) Calculation of enzyme activity :

$$U/ml = \frac{(A_1 - A_0)}{21.7} \times \frac{1}{10} \times \frac{3.02}{0.02} \times Z$$

wherein

21.7 : molecular absorption coefficiency cm^2/μ mol

Z : dilution ratio

Example 1

Reagent :

100 mM	Tris-HCl buffer (pH 9.5)
5 mM	Thio-NAD (Sankyo Co.)
0.2 mM	NADH (Oriental Yeast Co.)
92 U/ml	L-carnitine dehydrogenase (obtained from Ref. Example 2)

Operation :

The above reagent (1 ml) was put into cuvettes and L-carnitine solution (0.05 ml)(0, 10, 20, 30, 40 and 50 µM, respectively) was added therein, and started the reaction at 37 °C. After incubation was started, a difference in absorbancy at 3 mins. and 5 mins. was measured. Result is shown in Fig. 1. Linear realtion between an amonunt of L-carnitine and changes absorption was observed.

Example 2

Reagent :

40 mM	Glycine ·NaOH buffer (pH 10.0)

5 mM	Thio-NAD (Sankyo Co.)
0.5 mM	NADH (Oriental Yeast Co.)
0.5 %	polyoxyethylene (20) sorbitan monooleate (Wako Pure Chem. Co.)
120 U/ml	L-carnitine dehydrogenase (obtained from Ref. Example 2)
2 mM	Oxamic acid

Operation :

The above reagent (1 ml) was put into cuvette. Five-fold dilution of normal serum (each 50 µl) was added therein, and incubated at 37 °C. After reaction was started, absorbancy at 400 nm was measured at 1 min. and 6 min. Difference of absorbancy at 1 min. and 6 min. is shown in Fig. 2.

50 µM L-carnitine solution (50 µl) was also treated in the same manner as described above. Then L-carnitine in the normal serum was calculated and was observed as 54.3 µM.

Example 3

Reagent :

40 mM	Glycine-NaOH buffer (pH 10.0)
5 mM	Thio-NAD (Sankyo Co)
0.5 mM	NADH (Oriental Yeast Co.)
0.5 %	Polyoxyethylene (20) sorbitan monooleate (Wako Pure Chem. Co.)
120 U/ml	L-carnitine dehydrogenase (obtained from Ref. Example 2)
2 mM	Oxamic acid

Operation :

The above reagent (1 ml) was put into cuvettes. Specimen (50 µl), to which L-carnitine was added to normal serum in concentration of L-carnitine being 10, 20 and 50 µM, was treated in the same manner as described in Example 2. Result is shown in Table 1, where yield is observed as 97.0-102.0 %.

Table 1

Ammount added (µM)	Observed (µM)	Difference (µM)	Yield (%)
0	54.3	—	—
10	64.5	10.2	102.0
20	74.2	19.9	99.5
50	103.3	48.5	97.0

Example 4

Reagent :

40 mM	Glycine-NaOH buffer (pH 10.0)
5 mM	Thio-NAD (Sankyo Co.)
0.2 mM	NADH (Oriental Yeast Co.)
0.5 %	Polyoxyethylene (20) sorbitan monooleate (Wako Pure Chem. Co.)
100 U/ml	L-carnitine dehydrogenase (obtained from Ref. Example 2)
2 mM	Oxamic acid

Operation :

2 mM Tris-HCl buffer (0.025 ml) and 1 N KOH (0.025 ml) were added to serum (0.05 ml). Acylcarnitine in serum was hydrolysed by incubating at 37°C for one hour. Therefater the reaction mixture was neutralized by

adding 2.5 N HCl (0.05 ml) to prepare a specimen for total carnitine assay. A mixture of serum (0.05 ml) and physiological saline (0.1 ml) was prepared as a specimen for free carnitine assay.

The above each reagent (1 ml) was put into cuvettes, and a specimen hereinabove prepared (each 0.05 ml) was added to each cuvett respectively, then incubated at 37°C. After reaction was started, a difference at 1 min. and 6 min. in abosorption at 400 nm was obtained.

L-carnitine with known concentration was treated in the same manner as above, and an amount of total carnitine and that of free carnitine were calculated from the observed value, then an amount of acylcarnitine was measured from a difference between amount of total and free carnitine.

In Table 2, result obtained from three different serums is shown.

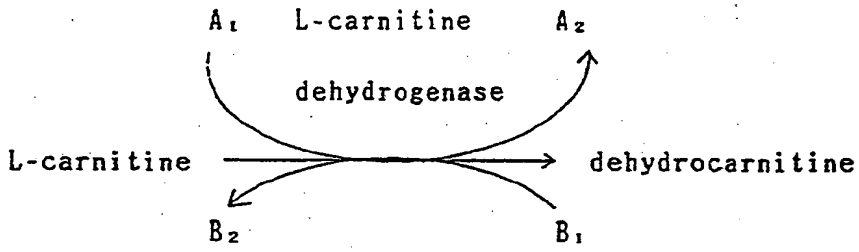
Table 2

	Total carnitine(μM)	Free carnitine(μM)	Acylcarnitine (μM)
Serum 1	84.5	35.3	49.2
Serum 2	50.0	26.3	23.7
Serum 3	89.5	43.2	46.3

Although the present invention has been described in connection with various preferred embodiments thereof, it will be appreciated that these embodiments are provided solely for purposes of illustration, and should not be construed as limiting the scope of the invention. Other embodiments and applications of the invention will be readily apparent to those skilled in the art from reading the present specification and practicing the techniques described herein, without departing whatsoever from the scope ans spirit of the appended claims.

Claims

1. A method of assaying L-carnitine which comprises reacting a specimen comprising the L-carnitine to be assayed with reagents comprising :
   ① L-carnitine dehydrogenase which has coenzymes of the thionicotinamide adenine dinucleotide group (hereinafter designated as the thio-NAD-group) and nicotinamide adenine dinucleotide group (hereinafter designated as the NAD group) and which catalyses a reversible reaction forming essentially dehydrocarnitine from L-carnitine ;
   ② A_1 ; and
   ③ B_1 ;
   to perform the cycling reaction :



wherein :
A_1 is the thio-NAD group or the NAD group ;
A_2 is a reduced form of A_1 ;
when A_1 is the thio-NAD group, B_1 is a reduced NAD group and when A_1 is the NAD group, B_1 is a reduced thio-NAD group, and
B_2 is an oxidized form of B_1 ;
and measuring the amount of A_2 generated or B_1 consumed.

2. A method according to claim 1 wherein the L-carnitine dehydrogenase has been obtained from the genus Alcaligenes.

3. A method according to claim 2 wherein the L-carnitine dehydrogenase has been obtained from Alcaligenes sp. No. 981 FERM BP-2570.

4. A method according to any one of the preceding claims wherein the NAD group is nicotinamide adenine dinucleotide (NAD), acetylpyridine adenine dinucleotide (acetyl NAD), acetylpyridine hypoxanthine dinucleotide or nicotinamide hypoxanthine dinucleotide (deamino-NAD).

5. A method according to any one of the preceding claims wherein the thio-NAD group is thionicotinamid adenine dinucleotide (thio-NAD) or thionicotinamide hypoxanthine dinucleotide.

6. A composition suitable for assaying L-carnitine which comprises components ① - ③ as defined in any one of the preceding claims.

7. L-carnitine dehydrogenase obtained from Alcaligenes sp. No. 981 FERM BP-2570.

8. Alcaligenes sp. No. 981 FERM BP-2570.

FIG. 1

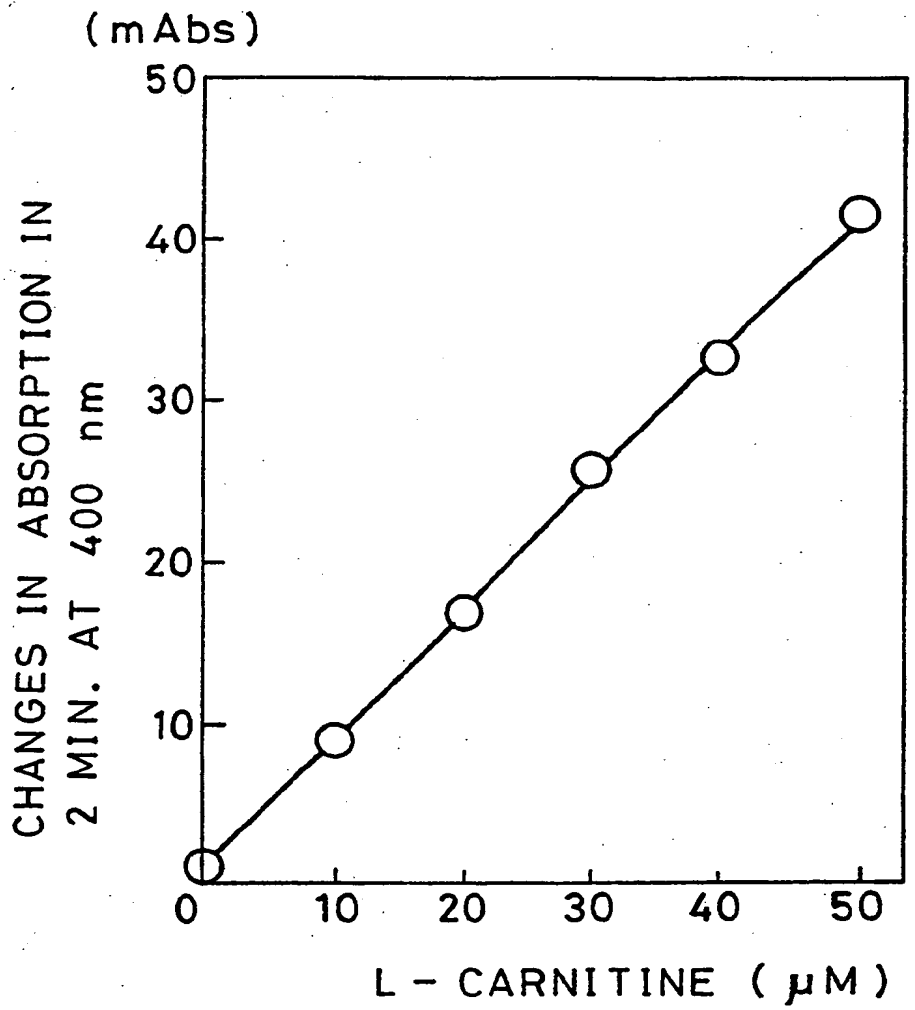
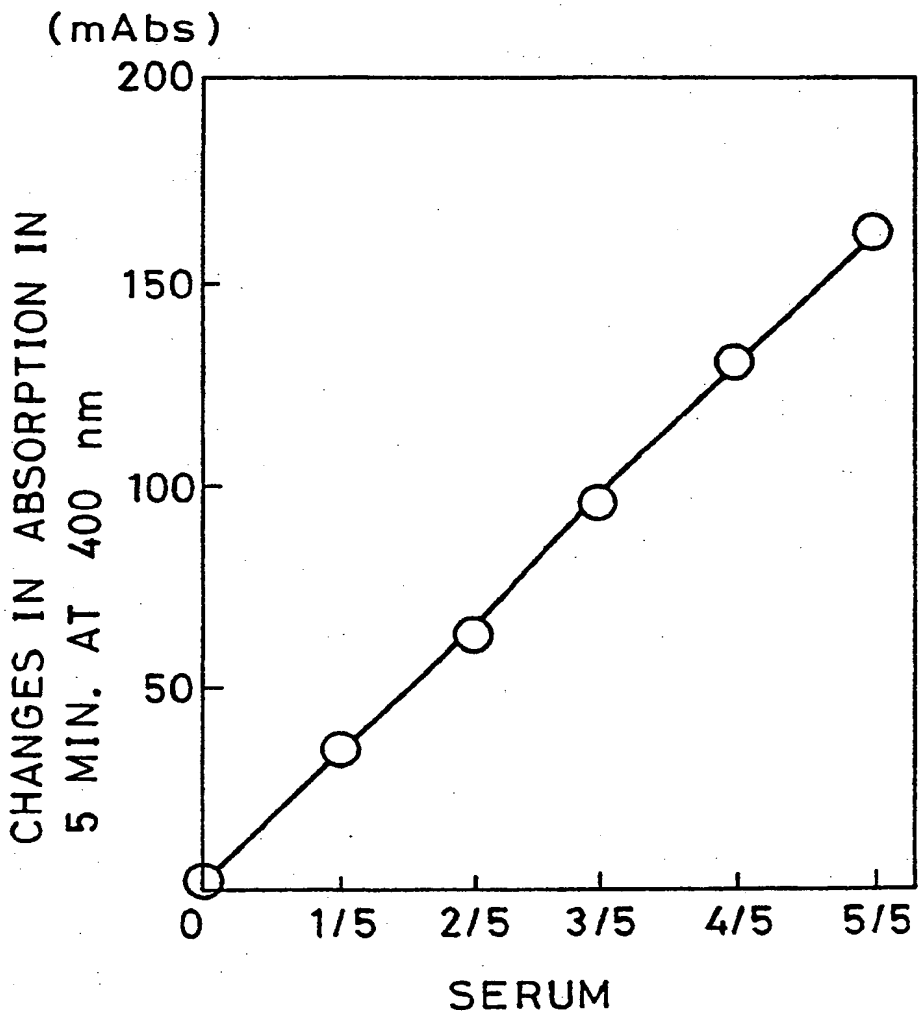


FIG. 2



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EUROPEAN SEARCH REPORT

Application Number

EP 91 30 0201

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	FR-A-2 398 046 (INSTITUT FRANCAIS DU PETROLE) * The entire document *	1,4-6	C 12 Q 1/26 C 12 Q 1/32 C 12 N 9/04 C 12 N 1/20 // (C 12 N 9/04 C 12 R 1:05)
A	---	2,3,7,8	
Y	FR-A-2 596 865 (SOCIETE NATIONALE ELF AQUITAINE) * Page 2, line 32 - page 6, line 14; claims 1-5 *	1,4-6	
D,Y	FRESENIUS' ZEITSCHRIFT FÜR ANALYTISCHE CHEMIE, vol. 320, no. 3, March 1985, pages 285-289, Springer Verlag, Berlin, DE; W. SCHÖPP et al.: "Quantitative Bestimmung von L-Carnitin mit Hilfe von Carnitindehydrogenase aus Pseudomonas putida" * The entire document *	1,4-6	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

C 12 Q
C 12 N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	13-03-1991	DOEPFER K-P.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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